Journal of Cellular Biochemistry 94:684–694 (2005)

Role of Thy-1 in In Vivo and In Vitro Neural Development and Regeneration of Dorsal Root Ganglionic Neurons

Chien-Hsing Chen,[1] Seu-Mei Wang,[1]* Shih-Hung Yang,[2] and Chung-Jiuan Jeng[3]**

[1]Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan
[2]Department of Surgery, National Taiwan University Hospital, Taiwan
[3]School of Medicine, Fu Jen Catholic University, Taipei Hsien, Taiwan

Abstract We have examined the expression of Thy-1, an abundant glycosylphosphatidylinositol (GPI)-anchored glycoprotein, in dorsal root ganglia (DRG) and associated nerve fascicles, during postnatal development and following a nerve crush. The expression levels of Thy-1 in DRG neurons, dorsal roots, and central processes in spinal cord were rather low at postnatal day 2, and gradually increased as DRG neurons matured. During early development, the expression of Thy-1 within DRG neurons was low and equally distributed between plasma membrane and cytosol. With maturation, the staining intensities of Thy-1 in both the plasma membrane and the cytosol of DRG neurons became increased. We also studied Thy-1 expression in the regeneration of mature DRG neurons following the crush injury of sciatic nerve. Two days after the crush injury, Thy-1 expression dramatically decreased in the DRG neurons on the lesion side. Between 4 and 7 days after the injury, the expression of Thy-1 gradually increased and returned to a normal level 1 week after the sciatic nerve crush. The time course of the up-regulation of Thy-1 expression during regeneration matched that of the recovery of sensory functions, such as pain withdraw reflex, placing reflex, and the score of Basso–Beattie–Bresnahan Locomotor Rating Scale. Taken together, our results suggest that Thy-1 expression is developmentally regulated and is closely associated with the functional maturation of DRG neurons during both postnatal development and nerve regeneration. Furthermore, perturbation of Thy-1 function with anti-Thy-1 antibodies promoted neurite outgrowth from primary cultured DRG neurons, again confirming the inhibitory role of Thy-1 on neurite outgrowth. J. Cell. Biochem. 94: 684–694, 2005.

Key words: Thy-1; DRG neurons; postnatal development; nerve injury; antibody perturbation

Thy-1, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein, is a major cell-surface molecule expressed most abundantly in the brain and thymus [Low and Kincade, 1985; Tse et al., 1985; Morris and Grosveld, 1989]. Thy-1 is also expressed in certain stages of lymphocytes, fibroblasts, endothelial cells, and glial cells; however, tissue distributions of Thy-1 differ among different animal species [Henniker, 2001; Barlow et al., 2002]. Thy-1 belongs to the immunoglobulin superfamily, which is a group of cell surface glycoproteins sharing structural motifs related to immunoglobulin and fibronectin domains [Walsh and Doherty, 1992]. Since most members of the immunoglobulin superfamily are adhesion molecules, it has been suggested that Thy-1 may also function as a cell adhesion molecule. For example, Thy-1 has been implicated in mediating the adhesion of thymocytes to thymic epithelial cells [He et al., 1991], in regulating thymocyte apoptosis [Hueber et al., 1994], and in modulating neurite outgrowth in neurons [Tiveron et al., 1992]. The most direct evidence of Thy-1 as an adhesion molecule comes from a

Grant sponsor: National Science Council, Republic of China; Grant number: NSC 92-2320-B-002-096; Grant sponsor: College of Medicine, Fu Jen Catholic University (internal fund).

*Correspondence to: Dr. Seu-Mei Wang, Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan.
E-mail: smwang@ha.mc.ntu.edu.tw

**Correspondence to: Dr. Chung-Jiuan Jeng, School of Medicine, Fu Jen Catholic University, 510 Chung-Cheng Road, Hsin-Chuang, Taipei Hsien, Taiwan 24205.
E-mail: jeng@mails.fju.edu.tw

Received 30 August 2004; Accepted 21 September 2004
DOI 10.1002/jcb.20341

recent identification of a Thy-1 ligand, glial β3 integrin, whose interaction with Thy-1 triggers tyrosine phosphorylation of focal adhesion proteins in astrocytes, thereby promoting adhesion of adjacent astrocytes to the underlying surface [Leyton et al., 2001; Avalos et al., 2002]. In addition to serving as a ligand, Thy-1 has also been implicated in signal transduction, possibly via increasing intracellular Ca^{2+} [Kroczek et al., 1986; Barboni et al., 1991] and stimulating tyrosine phosphorylation [Garnett et al., 1993]. Since Thy-1 is a GPI-anchored protein and does not possess either a classical transmembrane domain or a cytoplasmic portion, exactly how Thy-1 transduces activation signals in neurons remains elusive.

The expression of Thy-1 is mainly a postnatal event and is developmentally regulated [Xue and Morris, 1990; Barlow and Huntley, 2000]. During development, the immunoreactivity of Thy-1 is detected on the somatodendritic membrane of neurons [Xue et al., 1991]. However, Thy-1 expression is mostly excluded from regions where active axonal growth takes place [Xue et al., 1991; Morris et al., 1992]. Therefore, Thy-1 has been suggested to hinder the outgrowth of neurites and may play a negative role in neurite initiation, which is in fact supported by the observation from many in vitro functional studies. For example, it has been reported that neuronal differentiation and neurite outgrowth can be triggered by either cross-linking or removal of cell surface Thy-1 in both primary neurons and PC12 cells [Mahanthappa and Patterson, 1992a,b]. Likewise, expression of Thy-1 in a neuroblastoma cell line results in the inhibition of its neurite outgrowth on astrocytes [Tiveron et al., 1992], indicating that Thy-1 could downregulate the growth of axons in contact with astrocytes.

The functional significance of Thy-1 in neurons may be more than a negative regulator of neurite outgrowth. Thy-1 null mutant mice appear normal in neural development and neuronal architecture [Nosten-Bertrand et al., 1996; Barlow et al., 2002]. In these Thy-1 null mutant mice, however, there is a specific deficit in long-term potentiation in dentate gyrus of hippocampus [Nosten-Bertrand et al., 1996]. In addition, the mice display distinct defects in the test for social transmission of food preference [Mayeux-Portas et al., 2000]. These results suggest that Thy-1 may participate in the synaptic activity in the brain. The synaptic role of Thy-1 may involve synaptic transmission, since antibodies against Thy-1 can inhibit the regulated vesicular release of neurotransmitters [Jeng et al., 1998].

Most of the previous reports on the expression pattern of Thy-1 focus on studying the central nervous system (CNS). Since Thy-1 is an abundant and ubiquitous protein in the CNS, it is not easy to study the regulatory role of Thy-1 during axogenesis in a distinct cell type. The purpose of this study was to explore the expression pattern of Thy-1 in DRG neurons and associated nerve fascicles during the postnatal development and the regeneration process. Therefore, we used DRG neurons as an in vivo model system to investigate the expression and the potential function of Thy-1 in the development and regeneration of the sciatic nerve and DRG neurons. Addition of antibody to the culture medium perturbs Thy-1 function and effectively enhances neurite sprouting of superior cervical ganglionic neurons and PC12 cells [Mahanthappa and Patterson, 1992a,b]. This study also introduced antibody perturbation to approach the role of Thy-1 in neurite outgrowth of DRG neurons in vitro. Our results suggest that, during neural regeneration process, Thy-1 may recapitulate its inhibitory role on neurite outgrowth in developing neurons. The inhibitory function of Thy-1 on neurite outgrowth in DRG neurons was confirmed in both in vivo and in vitro models.

MATERIALS AND METHODS

Animals

Female Wistar rats were purchased from the Facility for Animal Research of the National Taiwan University. The date of the presence of vagina plug was considered as E0 and the date of birth was considered as postnatal day P0. For the developmental studies, postnatal animals ranging from 2-day-old (P2) to 8-week-old and 1-year-old animals were used and for regeneration experiments, 8-week-old rats were used. At least five animals were examined for each experimental group. Animal care and procedures were performed according to the standard set forth by the "Guide for the Care and the Use of Laboratory Animals," published by US National Institutes of Health (NIH publication N0. 85-23, revised 1985).

Sciatic Nerve Crush

Eight-week-old female Wistar rats were used for all the experiments. The left sciatic nerve was exposed at a mid-thigh level and crushed twice with a pair of aneurysm clip (250 g; holding force) for 20 s with a 15 s interval of relief. The wound was sutured in two layers of muscle and skin. The right sciatic nerve was left intact and served as the control group. After the surgery, animals were found to drag their injured hindlimb, but were able to move functionally around in their cage. Animals were allowed to recover for 2, 4, and 7 days after the surgery. Animal behaviors, including pain withdraw reflex, placing and BBB (Basso–Beattie–Bresnahan Locomotor Rating Scale) scores for locomotion [Basso et al., 1995], were examined every day until the animals were sacrificed.

Immunohistochemistry for Paraffin Sections

The animals to be used for immunohistochemistry studies were anesthetized with 7% chlorohydrate (0.45 ml/100 g body weight) and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. A dorsal laminectomy was performed to remove L4, L5, and L6 lumbar DRGs and spinal cords. The tissues were postfixed for 3 h in 4% paraformaldehyde. After the routine procedure of paraffin embedding, 7 μm thick sections were cut and mounted on silane-coated slides (Muto pure chemicals Co., Ltd., Japan). Tissue sections were deparaffinized, rehydrated in a series of alcohol, and immersed in 0.01M citrate buffer (pH 6.0). After antigen-retrieval in 0.01M citrate buffer by microwave, sections were blocked with 5% non-fat milk in phosphate-buffered saline (PBS), containing 0.1% Triton X-100 for 1 h. Sections were then incubated in primary antibody overnight at 4°C (1:500 diluted mouse anti-Thy-1 [Jeng et al., 1998], 1:500 diluted mouse anti-neurofilament 160 (NF160) (Sigma Chemical Co., St. Louis, MO), or 1:500 diluted mouse anti-neurofilament L (NF-L) (Sigma). After PBS wash, sections were incubated in FITC-conjugated goat anti-mouse IgG (Vector Labs, Burlingame, CA) at 1:50 dilution, washed with PBS and mounted with aqueous mounting medium (3% *n*-propyl gallate and 50% glycerol in PBS). For immunoperoxidase staining, after primary antibody incubation and PBS wash, some tissue sections were incubated in diluted peroxidase-conjugated goat anti-mouse IgG (Vector Labs). The peroxidase reaction was then visualized by developing with the SG substrate solution (Vector Labs). Images were taken on a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkocheu), equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan).

Western Blot

To measure protein expression, DRGs at lumbar 4–6 were surgically removed, homogenized in ice-cold lysis buffer solution (10 mM EGTA, 2 mM $MgCl_2$, 0.15% Triton X-100, 60 mM PIPES, 25 mM HEPES, pH 6.9, and 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 μg/ml of leupeptin, and 1 μg/ml pepstatin) and then sonicated. An equal volume of 2× reducing SDS sample buffer was added to the lysates and boiled at 95°C for 5 min. Protein samples (50 μg protein/gel lane) (protein determined by Bio-Rad protein Kit, Bio-Rad Lab, Hercules, CA) were separated on 10% polyacrylamide–SDS gels, electrotransferred to nitrocellulose filter (Schleicher and Schuell, Inc., Keene, NH), blocked by Tris-buffered saline (TBS: 50 mM Tris-Base, 150 mM NaCl, pH 8.2) containing 5% non-fat milk and 0.1% Tween-20, and then incubated overnight at 4°C in primary antibodies (mouse anti-Thy-1: 1:200; anti-β-actin: 1:1,000). Following washes with TBST (TBS containing 0.1% Tween-20), alkaline phosphatase conjugated secondary antibodies at 1:7,500 dilution (Promega corporation, Madison, WI) were added, and the bound antibodies were then visualized using enzyme-substrate reaction (Substrate: 3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-base, pH 9.5).

Quantification

Immunostained sections were photographed at 40× magnification, and the images were transferred into 256 gray scale images. Each experimental group consisted of five animals, and at least four sections from each animal were examined. The relative optical density of Thy-1 immunoreactivity was assessed, using a PC-based image analyzer software (Image Pro 3.0 Plus, Media cybernetics, Silver spring, MD) and those for Western blotting was quantified with Gel pro 3.1 (Media cybernetics). Only cells with intact nuclei were sampled, and DRG neurons

were classified into large ($\geq$40 μm) and small ($<$40 μm) according to the criteria described by Gavazzi et al. [2000]. The Western blots used for statistical analysis were repeated at least three times for each group. The density of the control group (8W animals) was defined as 100 and the densities of the other groups were expressed relatively. Student's *t*-test was used for evaluating statistical differences between the means of different groups, a *P*-value of less than 0.05 was considered significant.

Cell Culture

The DRG of P2 rats were dissected out and dissociated with 0.25% trypsin and 0.05% collagenase (Type I; Sigma) in Hank's balanced saline solution for 15 min at 37°C. Single cell culture was achieved by mechanical aspirating with a glass pipette, and collected by low-speed centrifugation. DRG neurons were cultured in a nutrient medium of L-15 Leibovitz medium (Gibco, Grand Island, NY), supplemented with 1.2 g/L of $NaHCO_3$, 5% fetal bovine serum, 100 IU/ml of penicillin and streptomycin, and maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were plated on grid-coverslips (Bellco Glass, Inc., Vineland, NJ) for neuron tracing.

Antibody Treatment

Ascites fluids of mouse anti-Thy-1 antibody were prepared according to Jeng et al. [1998], and were added to day-2 DRG cultures at a final dilution of 1:50 for 6 h. Normal mouse IgG (Sigma) was also applied to the culture at 1:50 dilution as the controls.

Tracing of Neurites

The same DRG neurons on the grid-coverslips were photographed using a Nikon inverted phase microscope at 0, 3, 6, 12, and 24 h. The total length of neurites radiated from a single neuron was calculated using Program Pro-plus, version 3.0 (Media Cybernetics).

Immunocytochemistry for Cultured DRG Neurons

After neuron tracing, DRG neurons on coverslips were fixed for 10 min with 10% formalin in PBS, treated with 0.15% Triton X-100 and 5% non-fat milk in PBS for 1 h, and incubated with monoclonal anti-NF-L antibody at 4°C overnight. After several washes in PBS, DRG neurons were reacted with FITC-conjugated goat anti-mouse IgG for 1 h at 37°C, washed in PBS and mounted in a mounting medium as described above.

RESULTS

Expression Pattern of Thy-1 in DRG Neurons During Development

Expression of Thy-1 at various time points during postnatal development was investigated by immunohistochemistry. We found that the expression level of Thy-1 gradually increased following the developmental course. At postnatal day 2 (P2), most DRG neurons were weakly positive for Thy-1 (Fig. 1A). At 1-week-old, the immunoreactivity of Thy-1 was diffusely distributed in the cytosol in most of the cell bodies of DRG neurons; furthermore, punctate staining was observed on the plasma membrane in some DRG neurons (Fig. 1B). During this early postnatal period, nerve fibers within DRG, as shown to be positive for NF-L (data not shown), were Thy-1-negative (Fig. 1A,B). About 50% of DRG neurons, including both large- and small-diameter neurons, were Thy-1-positive at 2-week-old (Fig. 1C). Punctate immunolabeling of Thy-1 was present on the plasma membrane of most DRG neurons. In addition, Thy-1 immunoreactive nerve fibers became visible within DRG (Fig. 1C). After 4 weeks of postnatal development, the immunoreactivity of Thy-1 in the cytosol and plasma membrane of DRG neurons substantially increased (Fig. 1D). At 8-week-old, Thy-1 was highly expressed in virtually all DRG neurons (Fig. 1E) and the immunoreactivity in both cytosol and plasma membrane significantly increased. At 1-year-old, the immunostaining of Thy-1 was also present in both the cytosol and the plasma membrane in neurons of all sizes, however, Thy-1 immunostaining was more localized to the plasma membrane in some of the large neurons (Fig. 1F). We then compared the immunoreactivity of Thy-1 with those of NF proteins, which includes NF-L and NF-160. NF-L is found to be the major neurofilament protein expressed in immature neurons, whereas NF-160 is the predominant form of NF expressed in mature neurons [Julien, 1999]. Therefore, we used NF-L immunolabeling as the internal standard for samples from P2 to 2-week-old, and NF-160 immunolabeling for samples from 4 weeks to 1-year-old. The immunoreactivity of NF-L was

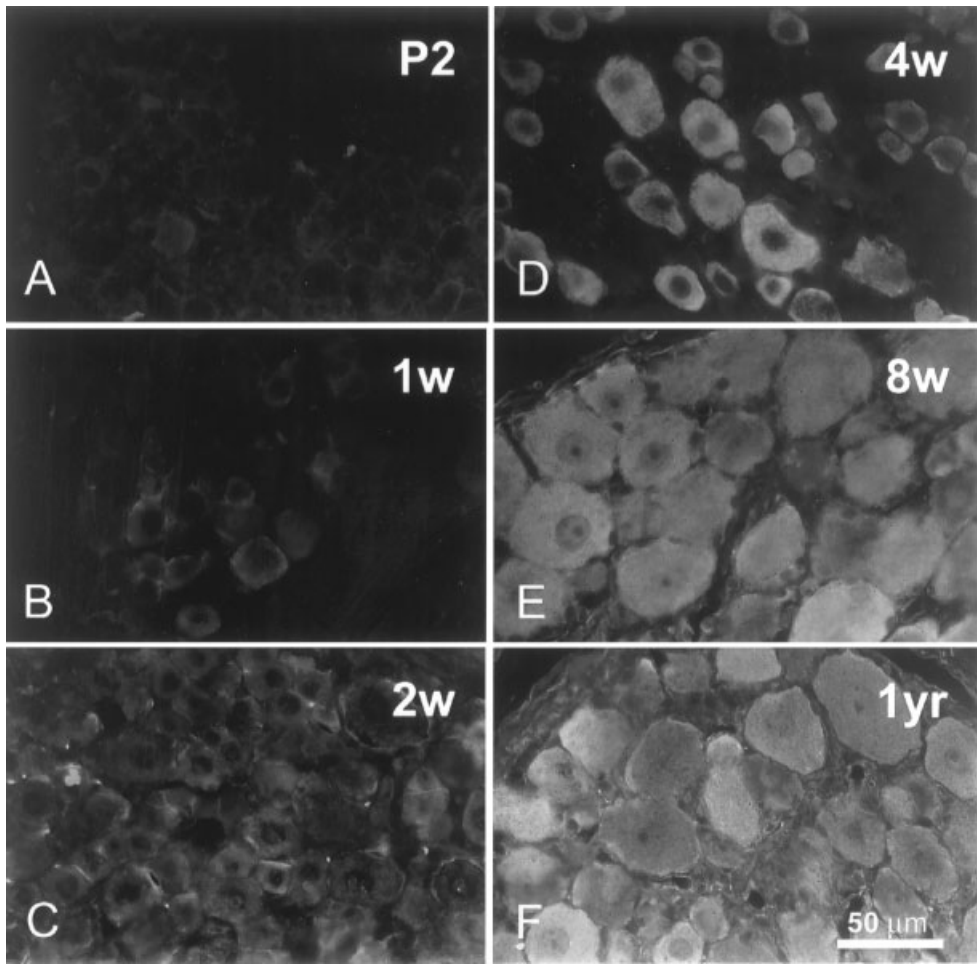

Fig. 1. Expression of Thy-1 in dorsal root ganglia (DRG) neurons during early postnatal development. **A**: At postnatal day 2 (P2), faint staining of Thy-1 immunolabeling appears on the plasma membrane of some neurons. **B**: At 1-week-old (1W), Thy-1 immunoreactivity is diffusely distributed in the cytosol and punctate staining is present on the plasma membrane of DRG neurons. **C**: At 2-week-old (2W), there is an increase of Thy-1 expression in DRG neurons. **D**: At 4-week-old (4W), the immunoreactivity of Thy-1 significantly increases. **E**: At 8-week-old (8W), Thy-1 immunolabeling is intensely expressed in all the DRG neurons. **F**: At 1-year-old (1 yr), Thy-1 immunoreactivity is detected in both the cytosol and the plasma membrane of small and large DRG neurons. Some large neurons only show Thy-1 staining on the plasma membrane. Bar represents 50 μm.

diffusely distributed in the cytosol of cell bodies and in nerve fibers within DRG as early as P2, and the staining intensity of NF-L gradually increased from 1-week-old (data not shown). With regard to the size of neurons, large-diameter neurons exhibited more NF-L immunolabeling than did small-diameter neurons. Similar to the staining pattern of NF-L, the immunoreactivity of NF-160 was widely distributed in the cytosol of cell bodies and in nerve fibers within DRG, the staining intensity of which was however down-regulated at 1-year-old (data not shown). We also examined the immunoreactivity of Thy-1 in spinal cords during early postnatal development. Thy-1 immunoreactive fibers failed to be detected at P2 and 1-week-old. Starting from 2-week-old, Thy-1-positive fibers were present in dorsal roots (data not shown) and dorsal columns (Fig. 2). Figure 2C showed that Thy-1-immunoreactive axons running into the dorsal horn. When the digital images of Thy-1 immunolabeling were converted into gray scale for optical density analysis, the results of optical density analysis closely matched the morphological observation. The optical densities of Thy-1 significantly increased from P2 to 8-week-old and reached a maximal level at 4-week-old (Fig. 3). There was about a 16-fold increase in the intensities of Thy-1 immunoreactivities in both large- and small-diameter neurons from P2 to 8-week-old.

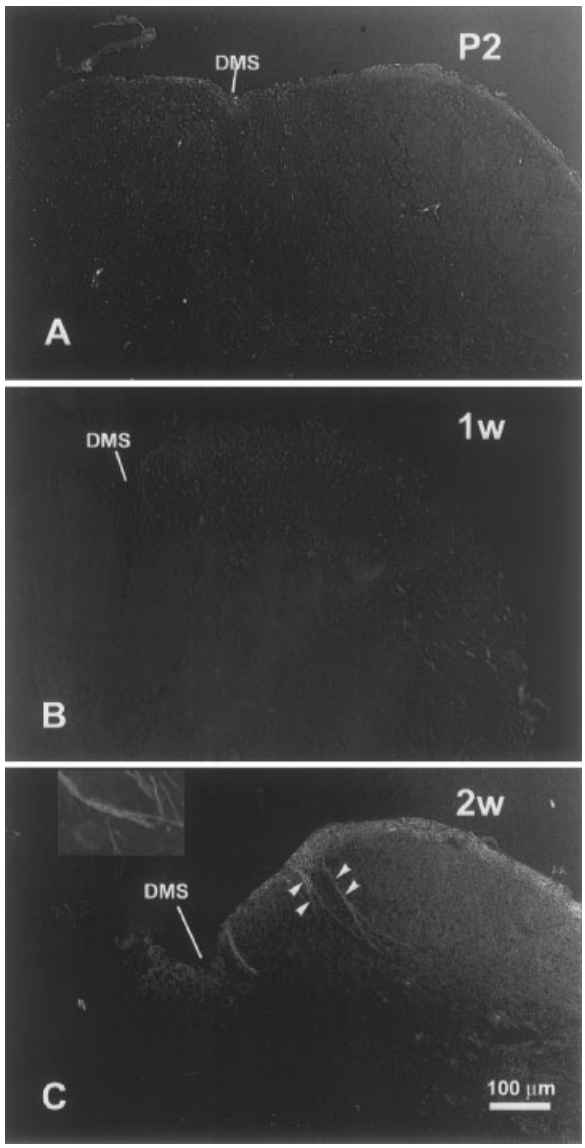

Fig. 2. Expression of Thy-1 in the spinal cord during early postnatal development. **A**, **B**, and **C**, are spinal cord sections obtained from animals of postnatal day 2, 1-, and 2-week-old. Arrowheads indicate the central processes of DRG neurons. Thy-1 immunoreactivity is detected by fluorescent labeling. DMS, dorsal median sulcus. Inlet: high magnification of Thy-1 positive fibers in C. Bar represents 100 μm.

The increase of Thy-1 expression during postnatal development was further confirmed by Western blot analysis. Starting from P2, there was a persistent increase of Thy-1 expression, which leveled at 2-week-old and stayed constant through 8-week-old (Fig. 4). In contrast, the expression level of β-actin remained at about the same level during the same developmental period (Fig. 4A).

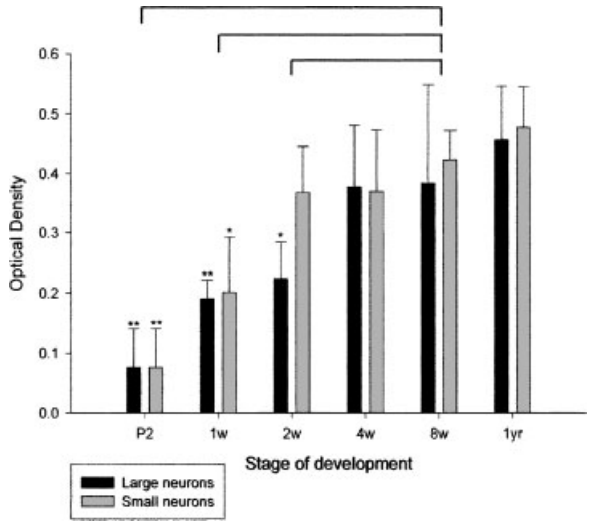

Fig. 3. Quantitative analysis of the immunostaining intensity of Thy-1 in DRG neurons during postnatal development. DRGs obtained from animals of different ages were immunostained for Thy-1 and the optical densities were quantitated as described in the materials and methods. *, $P < 0.05$; **, $P < 0.01$. (n = 3).

Expression Pattern of Thy-1 After Sciatic Nerve Injury

Next, we studied the expression of Thy-1 in DRG neurons during the regeneration process. Thy-1 immunoreactivity was assessed in rat lumbar DRG neurons at various time points

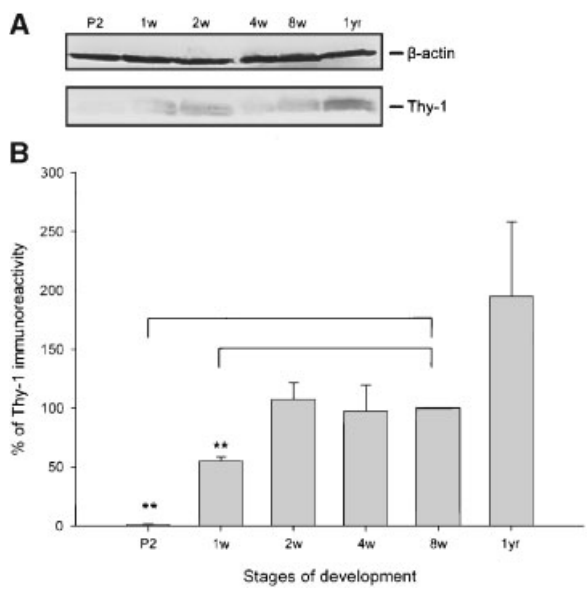

Fig. 4. Western blot analysis of Thy-1 expression in DRGs during postnatal development. **A**: DRGs obtained from animals at different ages as indicated were analyzed for Thy-1 and β-actin (an internal standard). Blot shown is representative of three separate experiments. **B**: Results of densitometric analysis of triplicate blots. **, $P < 0.01$ compared to the 8W group. (n = 3).

after the sciatic nerve crush. As shown in Figure 6, expression of Thy-1 dramatically decreased in the cytosol of large-diameter DRG neurons at 2 days (Fig. 5B) and 4 days (Fig. 5C) after the sciatic nerve crush. In control DRG neurons (Fig. 5A), Thy-1 immunoreactivity was observed in cytosol and plasma membrane of both large- and small-diameter neurons. By 2 days after the sciatic nerve crush, the intensity of Thy-1 immunoreactivity decreased markedly in the cytosol of large-diameter neurons; however, the punctate Thy-1 labeling on the plasma membrane was still obvious (Fig. 5B). At 4 days after the nerve crush, the intensity of Thy-1 immunoreactivity in the cytosol was still lower than that of the control group (Fig. 5C). At 7 days after the crush, Thy-1 immunoreactivity in the cytosol was increased and almost returned to the control level (Fig. 5D). There was no significant difference in the immunoreactivity of NF-160 in DRG neurons after the sciatic nerve crush (data not shown). Sciatic nerve stumps from the proximal and distal ends to the crush site were also collected and subjected to Thy-1 immunolabeling (data not shown). Thy-1 immunoreactivity was observed in the axonal membrane juxtaposed to the myelin membrane. The immunolabeling pattern in the proximal and distal stumps was not affected by the nerve crush. When analyzed for the optical density of Thy-1 immunoreactivity, large-diameter DRG neurons displayed a significant decrease of Thy-1 immunoreactivity at 2 and 4 days after the sciatic nerve crush (Fig. 6). In contrast, the intensity of Thy-1 immunoreactivity only slightly decreased in small-diameter DRG neurons by 4 days after the nerve crush. In addition to examining the change of Thy-1 immunoreactivity after the sciatic nerve injury, we also assessed the expression levels of Thy-1 in DRG neurons by Western blot analyses. The expression levels of Thy-1 decreased from 2 days after the nerve crush, reached the lowest level at 4 days, and returned to the normal levels at 1 week (Fig. 7).

The change of immunoreactivities and expression levels of Thy-1 in DRG neurons closely correlated with the alteration of motor and sensory functions as determined by the following behavioral tests. Right after the sciatic nerve crush, there was a loss of motor function (BBB score = 1), and both the pain withdrawal and the placing reflexes were absent. At 4 days after the lesion in the sciatic nerve, however, both motor and sensory function began to

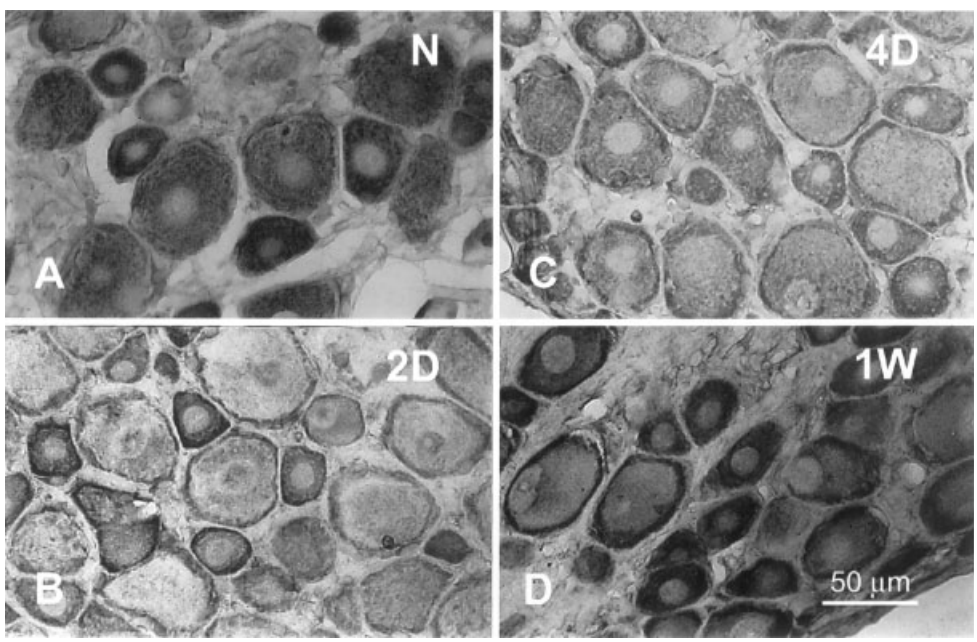

Fig. 5. Expression of Thy-1 in lumbar DRG neurons after the sciatic nerve crush. **A**: In small and large neurons of the control group (8-week-old rats), Thy-1 was intensely expressed both in the cytosol and the plasma membrane. **B**: At 2 days after the crush, the immunoreactivity of Thy-1 in large neurons significantly decreased. **C**: At 4 days after the crush, the immunostaining intensity of Thy-1 in large neurons was still lower than that of the control group. **D**: At 1 week after the crush, Thy-1 immunoreactivity in large neurons almost returned to a control level. Thy-1 immunoreactivity was visualized by peroxidase substrate. Bar represents 50 μm.

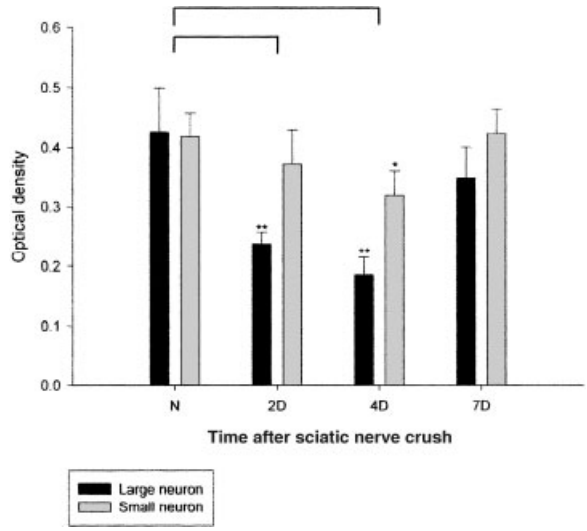

Fig. 6. Quantitative analysis of the immunostaining intensity of Thy-1 expression in lumbar DRGs after the sciatic nerve crush. DRGs obtained from various time points after the nerve crush were immunostained for Thy-1 and the optical densities were quantitated. Only large neurons show a fluctuation in Thy-1 immunoreactivity during recovery. *, $P < 0.05$; **, $P < 0.01$. (n = 3).

recover. The BBB score increased to nine, indicating the occurrence of reinnervation in the extensor muscles. About half of the animals investigated regained the pain withdraw reflex,

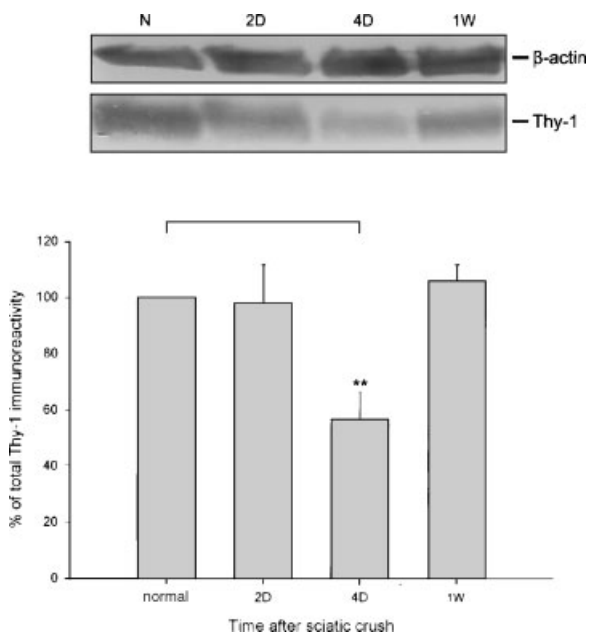

Fig. 7. Western blot analysis of Thy-1 expression in lumbar DRGs after the sciatic nerve crush. DRGs obtained from various time points after the nerve crush were analyzed for Thy-1 and β–actin expression. **, $P < 0.01$ compared to the 8W normal group. (n = 3).

but not the placing reflex, at 4 days after the lesion. The motor function and normal sensory reflexes recovered almost totally (BBB score = 21) at 1 week after the sciatic nerve crush.

Blockage of Thy-1 Function in Cultured DRG Neurons

The effects of Thy-1 antibody perturbation on DRG neurons were evaluated by both qualitative and quantitative ways. The elongation and arborization of the neurites were obviously increased in DRG neurons after treatment with anti-Thy-1 antibodies for 6 h (Fig. 8B), as compared with the controls (Fig. 8A). Figure 8B demonstrated the numerous neurites extended from a single neuron after anti-Thy-1 antibody treatment. In the control group, most of the neurons gave 2–4 primary branches, while in anti-Thy-1 group, the number of the primary branches increased to 3–6. After anti-Thy-1 treatment, the average total neurite length per cell increased about 2-fold of the control values (Fig. 8C).

DISCUSSION

The potential role of Thy-1 in the development and regeneration of peripheral sensory neuron was assessed in this study by investigating the expression pattern and the function of Thy-1 in DRG neurons both in vivo and in vitro systems. Our results support the negative regulatory role of Thy-1 in axonal growth.

Thy-1 has been suggested to be a negative regulator during neurite outgrowth, since it is mostly excluded from the regions of the active axonal growth [Xue et al., 1991; Morris et al., 1992]. In human embryonic DRG culture, Thy-1 expression is excluded from growing ganglionic axons by the 3rd week in culture and closely coupled with the formation of neurite fascicles [Almqvist et al., 1994]. Morphological studies have shown that many DRG neurons do not reach their final central targets until early postnatal stages [Fitzgerald, 1985; Snider et al., 1992]. Functional studies also indicate that the monosynaptic connection between dorsal root afferent axons and motor neurons has not yet completed at P5 [Snider et al., 1992], and that the C-fiber input to the dorsal horn does not fully develop until the second postnatal week [Fitzgerald and Gibson, 1984; Fitzgerald,

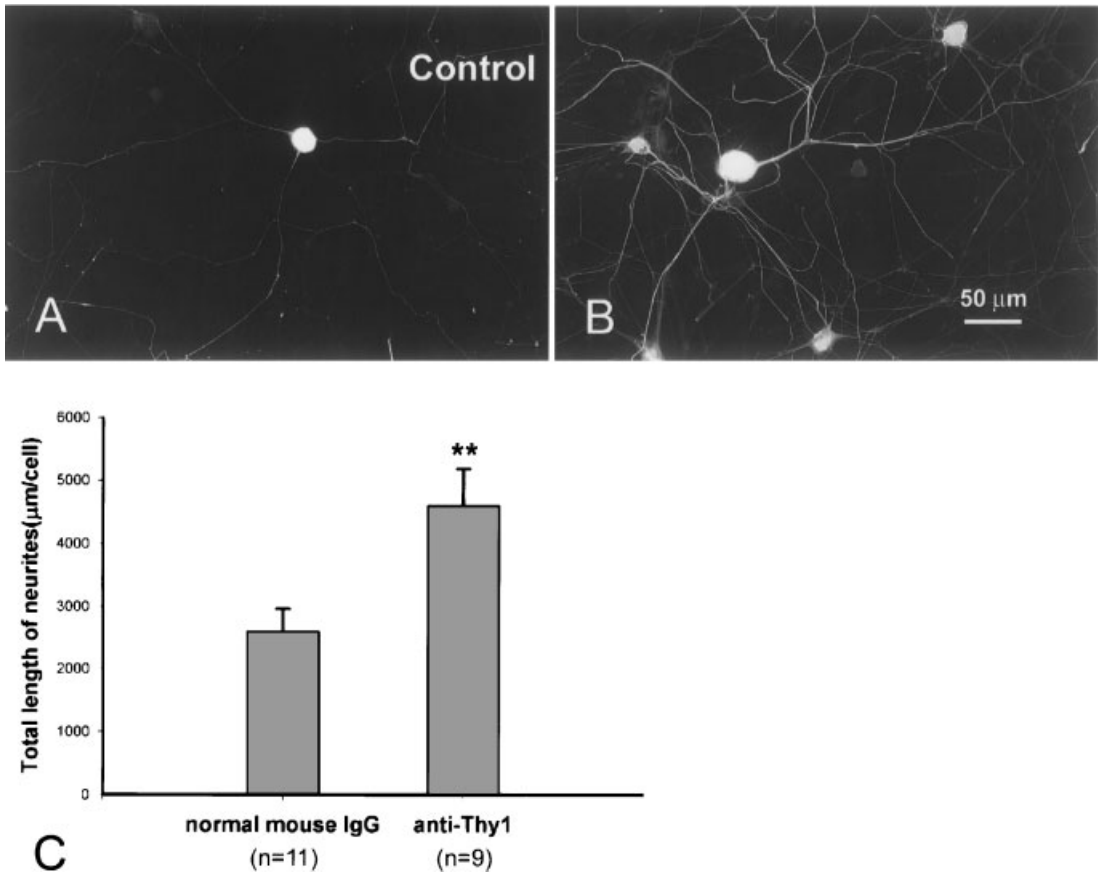

Fig. 8. Effect of anti-Thy-1 antibody perturbation on the growth pattern of cultured DRG neurons. DRG neurons were incubated with normal mouse IgG (control, **A**) and monoclonal anti-Thy-1 antibody (**B**) for 6 h, and then immunostained for NF-L. After 6 h of anti-Thy-1 incubation, the neurites were more elongated and branched (B) than those in the controls (A). Bar represents 50 μm. **C**: Quantitative analysis of the effect of antibody treatment on total neurite length per cell. N values for each group are indicated on the figure. **, $P < 0.01$.

1985]. Consistent with the previous observations, we found that the expression levels of Thy-1 in DRG neurons, dorsal roots, and dorsal columns were very low at P2. At 1-week-old, half of the DRG neurons expressed Thy-1 in the plasma membrane and cytosol; however, Thy-1-positive nerve fibers were not detectable in the dorsal root and dorsal column. Thy-1 expression both in DRG neurons and associated nerve bundles increased after the second postnatal week, and peaked at 8-weeks-old. Thus, our results support the idea that the time course of the central targeting of DRG neurons precedes that of the up-regulation of Thy-1 expression during development. Furthermore, we propose that after the targeting of DRG neurons is established, Thy-1 begins to express persistently on the plasma membrane, which may be essential to the regulation of the normal function of neurons. Although Thy-1 expression in large DRG neurons from 2W to 4W estimated by quantitative immunocytochemistry appeared to be increased, Thy-1 protein levels in 2W and 4W groups showed no significant difference by Western blot analysis. This discrepancy may be explained by the fact that in Western blot analysis, we actually analyzed Thy-1 protein from neurons of all sizes. Interestingly enough, Thy-1 expression level at 1-year-old was significantly higher than that at 8-week-old, suggesting that there was an up-regulation of Thy-1 expression after the maturation of neural development.

Our immunocytochemical analyses reveal that Thy-1 labeling in DRG neurons is equally distributed between the cytoplasm and the plasma membrane during the early development. As DRG neurons mature, Thy-1 expresses predominantly on plasma membrane in punctate distribution pattern, in contrast to the diffuse staining pattern of NF. The observation suggests that Thy-1 may be localized to

some microdomains, probably lipid rafts, on plasma membrane. Thy-1 has been shown to be located at lipid rafts in neuronal cells and thymocytes [Madore et al., 1999; Durrheim et al., 2001; Deininger et al., 2003]. Biochemical analyses suggest that Thy-1 is clustered with several Src-tyrosine kinases, such as fyn and lyn, in the detergent-insoluble lipid rafts [Stefanova et al., 1991; Draberova and Draber, 1993]. Further, Thy-1 forms complexes with tyrosine phosphatase in T cells, as shown by immunoprecipitation assay [Volarevic et al., 1990]. It will be interesting to investigate in the future the possibility of Src family kinases and tyrosine phosphates as the downstream signaling molecules mediating the negative regulatory effects of Thy-1 during postnatal development and neuronal regeneration.

While examining the sciatic nerve stumps both distal and proximal to the crush site, we did not observe a significant change in Thy-1 expression after the crush injury, which probably can be explained by the unusual slow turnover rate of Thy-1 [Lemansky et al., 1990]. The crush lesion applied in this study resulted in a mild damage which did not break the axonal membrane, but was able to disturb the synthesis of cytoskeletal proteins, such as β III-tubulin, the covering myelin sheath and surrounding connective tissues [Moskowitz and Oblinger, 1995]. Therefore, the damaged intramembranous structures and Schwann cells may still be able to trigger the alteration of Thy-1 expression pattern that we observed in DRG neurons.

We found in this study that Thy-1 expression is developmentally regulated in DRG neurons and associated nerve fascicles, and that the expression pattern of Thy-1 during development is temporally correlated with the time course of central targeting of DRG neurons. Moreover, based on the studies of the regeneration process of DRG neurons following a sciatic nerve crush, we propose that the developmental pattern of Thy-1 expression may be recapitulated after a peripheral nerve injury. In order to direct test the hypothesis that Thy-1 acts as a negative regulator on neurite outgrowth, we carried out the antibody perturbation experiment. Under this antibody treatment, DRG neurons exhibited longer neurite than the control neurons. Our data are consistent with the finding by Mahanthappa and Patterson [1992a] in cultured sympathetic neurons, PC12 cells, or adrenal chromaffin cells, which showed the addition of anti-Thy-1 antibody promotes neurite outgrowth. Similarly, removal of Thy-1 molecules from the cell surface by phosphatidylinositol-specific phospholipase C also abolishes the inhibitory effect of Thy-1 on neurite growth [Mahanthappa and Patterson, 1992a]. They hypothesized that the removal of Thy-1 from the cell surface might alter the regulation of some secondary messengers involved in neurite outgrowth, though other mechanisms may be involved. For example, cross-linking of Thy-1 by anti-Thy-1antibody increases the levels of intracellular calcium in T lymphocytes [Kroczek et al., 1986; Ledbetter et al., 1987] and elicits a transient calcium current across the membrane of DRG neurons [Saleh et al., 1988]. Thus, anti-Thy-1 may cross-link Thy-1 molecules, and this multimerization triggers downstream signaling associated with neurite outgrowth as proposed for PC12 cells [Mahanthappa and Patterson, 1992b] should be considered. Taken together, our data support the role of Thy-1 in negative regulation in neurite outgrowth both in vivo and in vitro systems.

REFERENCES

Almqvist P, Pschera H, Samuelsson EB, Lunell NO, Seiger A. 1994. Morphology and growth of embryonic, human dorsal root ganglion explants in long-term culture: Expression of cell type-specific markers during early differentiation. Exp Neurol 125:1–14.

Avalos AM, Labra CV, Quest AF, Leyton L. 2002. Signaling triggered by Thy-1 interaction with beta 3 integrin on astrocytes is an essential step towards unraveling neuronal Thy-1 function. Biol Res 35:231–238.

Barboni E, Gormley AM, Pliego Rivero FB, Vidal M, Morris RJ. 1991. Activation of T lymphocytes by cross-linking of glycophospholipid-anchored Thy-1 mobilizes separate pools of intracellular second messengers to those induced by the antigen-receptor/CD3 complex. Immunology 72: 457–463.

Barlow JZ, Huntley GW. 2000. Developmentally regulated expression of Thy-1 in structures of the mouse sensory-motor system. J Comp Neurol 421:215–233.

Barlow JZ, Kelley KA, Bozdagi O, Huntley GW. 2002. Testing the role of the cell-surface molecule Thy-1 in regeneration and plasticity of connectivity in the CNS. Neuroscience 111:837–852.

Basso DM, Beattie MS, Bresnahan JC. 1995. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 12:1–21.

Deininger SO, Rajendran L, Lottspeich F, Przybylski M, Illges H, Stuermer CA, Reuter A. 2003. Identification of teleost Thy-1 and association with the microdomain/lipid raft reggie proteins in regenerating CNS axons. Mol Cell Neurosci 22:544–554.

Draberova L, Draber P. 1993. Thy-1 glycoprotein and src-like protein-tyrosine kinase p53/p56lyn are associated in large detergent-resistant complexes in rat basophilic leukemia cells. Proc Natl Acad Sci USA 90:3611–3615.

Durrheim GA, Garnett D, Dennehy KM, Beyers AD. 2001. Thy-1 associated pp85–90 is a potential docking site for SH2 domain-containing signal transduction molecules. Cell Biol Int 25:33–42.

Fitzgerald M. 1985. The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. J Physiol 364:1–18.

Fitzgerald M, Gibson S. 1984. The postnatal physiological and neurochemical development of peripheral sensory C fibres. Neuroscience 13:933–944.

Garnett D, Barclay AN, Carmo AM, Beyers AD. 1993. The association of the protein tyrosine kinases p56lck and p60fyn with the glycosyl phosphatidylinositol-anchored proteins Thy-1 and CD48 in rat thymocytes is dependent on the state of cellular activation. Eur J Immunol 23: 2540–2544.

Gavazzi I, Stonehouse J, Sandvig A, Reza JN, Appiah-Kubi LS, Keynes R, Cohen J. 2000. Peripheral, but not central, axotomy induces neuropilin-1 mRNA expression in adult large diameter primary sensory neurons. J Comp Neurol 423:492–499.

He HT, Naquet P, Caillol D, Pierres M. 1991. Thy-1 supports adhesion of mouse thymocytes to thymic epithelial cells through a Ca^{2+}-independent mechanism. J Exp Med 173:515–518.

Henniker AJ. 2001. CD90. J Biol Regul Homeost Agents 15:392–393.

Hueber AO, Raposo G, Pierres M, He HT. 1994. Thy-1 triggers mouse thymocyte apoptosis through a bcl-2-resistant mechanism. J Exp Med 179:785–796.

Jeng CJ, McCarroll SA, Martin TF, Floor E, Adams J, Krantz D, Butz S, Edwards R, Schweitzer ES. 1998. Thy-1 is a component common to multiple populations of synaptic vesicles. J Cell Biol 140:685–698.

Julien JP. 1999. Neurofilament functions in health and disease. Curr Opin Neurobiol 9:554–560.

Kroczek RA, Gunter KC, Germain RN, Shevach EM. 1986. Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. Nature 322: 181–184.

Ledbetter JA, June CH, Grosmaire LS, Rabinovitch PS. 1987. Crosslinking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. Proc Natl Acad Sci USA 84:1384–1388.

Lemansky P, Fatemi SH, Gorican B, Meyale S, Rossero R, Tartakoff AM. 1990. Dynamics and longevity of the glycolipid-anchored membrane protein, Thy-1. J Cell Biol 110:1525–1531.

Leyton L, Schneider P, Labra CV, Ruegg C, Hetz CA, Quest AF, Bron C. 2001. Thy-1 binds to integrin beta(3) on astrocytes and triggers formation of focal contact sites. Curr Biol 11:1028–1038.

Low MG, Kincade PW. 1985. Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. Nature 318:62–64.

Madore N, Smith KL, Graham CH, Jen A, Brady K, Hall S, Morris R. 1999. Functionally different GPI proteins are organized in different domains on the neuronal surface. EMBO J 18:6917–6926.

Mahanthappa NK, Patterson PH. 1992a. Thy-1 involvement in neurite outgrowth: perturbation by antibodies, phospholipase C, and mutation. Dev Biol 150: 47–59.

Mahanthappa NK, Patterson PH. 1992b. Thy-1 multimerization is correlated with neurite outgrowth. Dev Biol 150:60–71.

Mayeux-Portas V, File SE, Stewart CL, Morris RJ. 2000. Mice lacking the cell adhesion molecule Thy-1 fail to use socially transmitted cues to direct their choice of food. Curr Biol 10:68–75.

Morris R, Grosveld F. 1989. Expression of Thy-1 in the nervous system of the rat and mouse. Immunol Ser 45: 121–148.

Morris RJ, Tiveron MC, Xue GP. 1992. The relation of the expression and function of the neuronal glycoprotein Thy-1 to axonal growth. Biochem Soc Trans 20:401–405.

Moskowitz PF, Oblinger MM. 1995. Sensory neurons selectively upregulate synthesis and transport of the beta III-tubulin protein during axonal regeneration. J Neurosci 15:1545–1555.

Nosten-Bertrand M, Errington ML, Murphy KP, Tokugawa Y, Barboni E, Kozlova E, Michalovich D, Morris RG, Silver J, Stewart CL, Bliss TV, Morris RJ. 1996. Normal spatial learning despite regional inhibition of LTP in mice lacking Thy-1. Nature 379:826–829.

Saleh M, Lang RJ, Bartlett PF. 1988. Thy-1-mediated regulation of a low-threshold transient calcium current in cultured sensory neurons. Proc Natl Acad Sci USA 85: 4543–4547.

Snider WD, Zhang L, Yusoof S, Gorukanti N, Tsering C. 1992. Interactions between dorsal root axons and their target motor neurons in developing mammalian spinal cord. J Neurosci 12:3494–3508.

Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 254: 1016–1019.

Tiveron MC, Barboni E, Pliego Rivero FB, Gormley AM, Seeley PJ, Grosveld F, Morris R. 1992. Selective inhibition of neurite outgrowth on mature astrocytes by Thy-1 glycoprotein. Nature 355:745–748.

Tse AG, Barclay AN, Watts A, Williams AF. 1985. A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. Science 230:1003–1008.

Volarevic S, Burns CM, Sussman JJ, Ashwell JD. 1990. Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. Proc Natl Acad Sci USA 87:7085–7089.

Walsh FS, Doherty P. 1992. Second messengers underlying cell-contact-dependent axonal growth stimulated by transfected N-CAM, N-cadherin, or L1. Cold Spring Harb Symp Quant Biol 57:431–440.

Xue GP, Morris RJ. 1990. Evidence for cell-type differences in the regulation of neuronal expression of Thy-1. Biochem Soc Trans 18:441–442.

Xue GP, Rivero BP, Morris RJ. 1991. The surface glycoprotein Thy-1 is excluded from growing axons during development: A study of the expression of Thy-1 during axogenesis in hippocampus and hindbrain. Development 112:161–176.